

**The Presence of Aromatase mRNA and Protein in Ovine Mammary Fat Pad and Possible  
Effect of Birth Status on its Abundance**

An Undergraduate Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduating with Honors at The Ohio  
State University

By

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Undergraduate Program in Animal Sciences

The Ohio State University  
2012

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## **Abstract**

Aromatase is an enzyme that is important to concentrations of sex hormone in both developing and mature animals. It converts the male sex-hormone testosterone to estrogen, the female sex-hormone. One of the many functions of estrogen is to stimulate the growth of mammary glands of prepubertal animals. A previous study conducted in the Animal Sciences Department at The Ohio State University provided detailed statistics about mammary gland and carcass traits of female lambs born with a twin. It seems that growth of mammary glands, specifically the fatty portion of the tissue, is influenced by the sex of the twin that is gestated with a female lamb. Female lambs with a male twin had 30% more fatty tissue in mammary glands than female lambs with a female twin. Another difference observed was that females with a male twin have less body fat than females with a female twin. Fat is one source of estrogen, so aromatase presence in the fatty tissue of female sheep mammary glands may be of developmental significance. We hypothesized that aromatase is present in the fatty tissue, and we further rationalized that the relative amount would be different depending on if female lambs were gestated with a female lamb or a male lamb. To test this, mammary tissue from 8 sets of female-female twin pairs (FF;  $n = 16$ ) and 11 ewe lambs gestated with a male (FM;  $n = 11$ ) was obtained and subjected to quantitative reverse transcriptase PCR and immunohistochemistry for the aromatase gene and protein, respectively. The aromatase gene was expressed in mammary parenchyma at low levels and was not different due to twin status ( $P = 0.267$ ); aromatase gene in the fatty portion was below the limit of detection for our assay. Aromatase was found through antibody staining to be primarily located in mammary epithelial cells in the parenchyma, and was found sparsely in the mammary fat pad. Importantly, we demonstrated aromatase at both the gene and protein levels in ewe lambs, which is a novel finding. This perhaps suggests that

aromatase is important for prepubertal mammary growth, though not in the manner we originally hypothesized.

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## **A. Introduction**

During normal mammary development in sheep, the mammary fat pad is remodeled during pregnancy and essentially vanishes before lactation. This transformation of fat pad to epithelial cells is crucial for proper milk production during lactation. A study conducted by Avdi and Driancourt (1997) compared the first lactation milk yield in sheep to what is known as their twin status. Twin status, as it will be called for the purposes of this study, refers to the gender of the twins; specifically here, the gender of the twin born with a female. The study indicated that milk yield is not affected by twin status. Despite this finding, twin status appeared to impart some changes in mammary composition that may still be of developmental significance (see Part B, Preliminary Data). The likely effector is testosterone, indirectly.

While testosterone is not present in high quantities postnatally in females, testosterone would be present in utero if a male twin was present. Exposure to testosterone and other androgens in utero can have lasting effects on these females by altering their sexual differentiation via a process known as prenatal androgenization. Hansen and others (1995a, 1995b) showed alterations in growth, carcass characteristics, blood metabolites, and liver and adipose metabolism in ewe lambs that were exposed to exogenous testosterone in utero, where development of androgenized ewe lambs were more similar to control wethers than to control ewes. This suggests that the intrauterine environment plays a role in future development of the ewe lamb (Hansen et al., 1995a, 1995b).

One interesting finding in androgenized ewe lambs is that they develop enlarged livers (DeHanan et al., 1987; Jenkins et al., 1988). It is thought that this contributes to the observed altered growth patterns due to the large amount of metabolic activity in the liver, fat catabolism

included. It was recently found that the liver is a catabolic site for testosterone too (Sharma et al., 2004). In the liver, testosterone was converted into estrogen in sufficient quantities to be of biological importance in male sheep (Sharma et al., 2004). Aromatase is the rate-limiting enzyme responsible for converting testosterone into estrogen; it is present in large quantities in the liver of male sheep (Sharma et al., 2004). Given the combination of large organ size and high aromatase activity, Sharma and colleagues called the liver the most important source of aromatase, and therefore estrogen, in the body of male sheep (Sharma et al., 2004).

Studying the mammary fat pad (MFP) is important in researching mammary growth because in addition to being an energy storage site, adipose is estrogenic. Estrogen is an important regulator of mammary development. In prepubertal animals, the MFP is thought to direct the development of the underlying epithelial tissue through the actions of estrogen. Despite the MFP being almost 30% larger in female ewe lambs gestated with males (FM), there was no proportional increase in the amount of epithelial tissue. This hints at a potentially suppressive effect of the MFP in this instance. The main source of estrogen in mature females is the ovary, but in prepubertal animals such as those used in this research, the main source of estrogen is from the conversion of estrogenic precursors in fat. One of the main estrogenic precursors is testosterone.

Testosterone is classically implicated in the development of secondary sex characteristics in males, such as the development of a lean body type through the catabolism of stored body fat. In sheep, fat deposition occurs earlier and faster in female lambs than in ram or castrated (whether) lambs (Hansen et al., 1995a.), causing ewe lambs to typically produce carcasses containing more fat than male counterparts.



The liver or local fat deposits are likely candidates for the main endogenous source of prepubertal lambs. We initially believed there was a link between the adiposity and size of the MFP and the level of activity of aromatase, which may be influenced by the latent effects of testosterone in FM twins. In partial support of this, both male and female knockout mice missing the gene coding for aromatase were not only unable synthesize endogenous estrogen but their intra-abdominal adipose tissue mass increased (Jones et al., 2000).

There is an apparent link between the presence of prenatal, in utero testosterone and the composition of the mammary gland of females born with a male twin. We hypothesized that aromatase might be this link.

## **B. Preliminary Data**

An unpublished study was conducted at the Animal Sciences Department at The Ohio State University to test for a correlation between the body condition score of a ewe during her pregnancy, and the mammary composition of her female offspring. While this study did not provide data that concluded that there is such a correlation, a review of the data collected on the female offspring revealed an interesting pattern. As shown in **Table 1**, it appears that the mammary gland and carcass composition of the female ewe lambs varies depending on the sex of her twin (that is, her twin status). The FM twins had MFP that were almost 30% larger and contained more total lipid than their female-female twin (FF) counterparts. FM ewe lambs were also found to have leaner carcasses compared to FF ewe lambs. These are seemingly at odds with each other and we speculate that these differences may have all arisen due to aromatase activity. This could mean that, even to a small degree, the female lambs with a male co-twin were androgenized in utero and that affected their subsequent development.

### **C. Problem Identification and Justification**

Our preliminary data in **Table 1** for the mammary and body composition of the test lambs generally agrees with the studies done by Hansen and others (1995a; 1995b), suggesting that the presence of testosterone in the intrauterine environment due to the presence of a ram twin may latently affect mammary development and composition. Testosterone is not implicated in mammary development but estrogen is. Aromatase is a rate-limiting enzyme involved in the conversion of testosterone to estrogen. It is suspected that aromatase may be involved in the observed adiposity of MFP in FM due to its known role in converting testosterone into estrogen and its observed effects on adiposity. Aromatase has been found at the gene and protein level in MFP of Holstein heifers (Huderson, 2009), but to our knowledge, these have not been studied in sheep.

### **D. Hypothesis and Objectives**

We hypothesized that mRNA for aromatase and its mature protein product would be found in the MFP of all lambs analyzed. We further hypothesized that aromatase mRNA and protein abundance would be different in FF twins compared to FM twins.

Our objectives were to:

- 1) To demonstrate aromatase mRNA presence and then quantify relative abundance in snap-frozen mammary tissue samples collected from females of FF and FM twin pairs born to first parity ewes.
- 2) To demonstrate aromatase protein presence and then quantify staining intensity in formalin-fixed, paraffin-embedded mammary tissue samples collected from the same lambs as noted above.

- 3) To ascertain if the level of expression of the aromatase gene correlates with that of the protein.

## **E. Materials and Methods**

*Tissue Samples:* The samples used here are a subset of samples collected from a larger trial recently conducted at The Ohio State University (Boesche et al., 2010). We intended to focus only on ewe lambs born to 1<sup>st</sup> parity ewes. Of these lambs, there were 8 sets of FF twins (n=16) and 11 FM ewe lambs. Samples of MFP and PAR from these 27 ewe lambs were taken at slaughter. Briefly, the whole udder was removed, weighed, and bisected along the median suspensory ligament. The left mammary gland was subsampled to obtain PAR and MFP for later RNA and histological analysis. Samples for RNA analysis were snap frozen in liquid nitrogen and stored at -80 °C until use. Samples for histology were fixed in formalin for 24h and stored in 70% EtOH for later paraffin-embedding and immunohistochemistry.

*Total RNA Extraction and cDNA Synthesis:* Total RNA from the snap-frozen MFP and PAR were extracted using RNeasy mini kits (Qiagen; Valencia, CA). The procedures used have recently been described by Rinaldi et al. (2010). RNA quality was verified on an Agilent Bioanalyzer 2100 (Agilent Technologies; Palo Alto, CA) and total RNA concentration was determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE). Total RNA yield was low for all MFP samples and averaged 1.85 µg total RNA/sample (n = 17), as calculated from NanoDrop readings. Total RNA from PAR samples, in contrast, averaged 29.75 µg total RNA/sample (n = 27). Because of this, MFP samples were subjected to an acid-ethanol RNA precipitation procedure in an attempt to concentrate the total RNA before reverse transcription. Briefly, samples with an initial volume of 25µl underwent the following

procedure. First, 2.5  $\mu$ l of 3M sodium acetate (pH 5.2) were added to each sample. Second, 62.5  $\mu$ l of 100% EtOH were added. Third, samples were vortexed and incubated at -80°C for at least 1h. Fourth, samples were centrifuged at 12,000 x g for 10 min at 4°C. Fifth, the supernatant was poured off and each pellet was washed with 90 $\mu$ l of 70% EtOH. Sixth, tubes were vortexed and centrifuged as in step four. Seventh, EtOH was removed and tubes were allowed to air dry for several minutes. Eighth, precipitated total RNA was resuspended by adding either 3.5  $\mu$ l (if tripling original MFP samples) or 5.5  $\mu$ l (if doubling original MFP samples) of nuclease-free water. Lastly, samples were denatured by heating at 65°C for 5 min. RNA concentrations of resuspended samples were rechecked on the NanoDrop. After RNA precipitation, yield of total RNA in MFP samples was still low and averaged 1.25  $\mu$ g total RNA/ sample (n = 26). Despite this, total RNA yield and quality in PAR and MFP were appropriate for further use in reverse transcription reactions.

Reverse transcription of PAR and MFP samples was carried out using an iScript cDNA Synthesis Kit (BioRad; Hercules, CA). Single-stranded cDNA was reverse transcribed from total RNA by denaturing 0.5  $\mu$ g of RNA in DNase/RNase free water at a final volume of 11.3  $\mu$ l at 70°C for 12 min. Samples were placed on ice for 3 min. Then, reverse transcription master mix (8.7  $\mu$ l) was made; it contained: 4  $\mu$ l of M-MLV RT 5x buffer (Promega; Madison, WI), 2  $\mu$ l of 0.1 M dithiothreitol, 1  $\mu$ l 10 mM dNTP mix (Promega; Madison, WI), 1  $\mu$ l of oglio(dT)15 primer, 0.5  $\mu$ l M-MLV reverse transcriptase (Promega; Madison, WI), and 0.2  $\mu$ l of RNase inhibitor (Promega; Madison, WI). The entire volume of master mix was added to each well of denatured RNA in a 96 well PCR plate for a final reaction volume of 20  $\mu$ l (Bio-Rad; Hercules, CA). Reverse transcription was carried out in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad; Hercules, CA) with cycle conditions at 40°C for 1 h, followed by 95°C for 10

min. Resultant cDNA was then diluted 1:1 with addition of 20 µl of DNase/RNase free water. cDNA was stored at -20°C until further use.

*Primer Design and Primer Efficiency:* Primers were designed with Primer3 (v. 0.4.0) for the aromatase gene as well as the endogenous control gene β-actin. Primer locations within the mRNA sequence for aromatase and the corresponding PCR product size (151 base pairs) are shown in (**Figure 1**). β-actin primers were as follows: forward, ACT-GGG-ACG-ACA-TGG-AGA-AG; reverse, GGG-GTG-TTG-AAG-GTC-TCA-AA. The designed β-actin PCR product size was 157 base pairs. Primer efficiency was determined for aromatase primers by the use of serial dilutions of cDNA, using the following equation: percent efficiency =  $(10^{-1/\text{slope}} - 1) * 100$ . Primer efficiency was 135%.

*Quantitative Reverse Transcription-PCR:* Quantitative reverse transcription-PCR (**qPCR**) reactions were carried out using a master mix consisting of: 10.5 µl of RNase/Dnase free water, 12.5 µl iQ SYBR Green Supermix (Bio-Rad; Hercules, CA), and 0.5 µl of each respective forward and reverse primer. 2 µl of master mix were then added to 2 µl of first-strand cDNA, yielding a 26 µl reaction volume for qPCR. Amplification was carried out in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad; Hercules, CA) with conditions set at: 1 cycle at 95°C for 5 min, 40 repeating cycles at 95°C for 10 s then at 55°C for 35 s (**Figure 2**). Immediately after the qPCR experiment a melt curve analysis was conducted to verify purity of amplicons and absence of primer dimers. For this, plate temperature started at 65°C and then increased to 95°C in 1°C increments (10 s per increment; **Figure 2**). Representative samples of PCR products for each primer pair were electrophoresed on a 2% agarose gel using the methodology of Swank (2012). This was to confirm purity and size of each amplicon (**Figure 3**).

*Relative Gene Expression Calculations:* Relative mRNA abundance of aromatase is expressed as fold difference relative to the expression of the endogenous reference gene  $\beta$ -actin. Calculations were as reported in Velayudhan et al. (2008). Data are presented as  $2^{(-\Delta Ct)}$ .  $\beta$ -actin was determined to be a worthy housekeeping gene because of its precise amplification within 2 cycles among mammary tissue samples of all calves (data not shown).

*Immunohistochemistry:* At slaughter, mammary interface samples (contain outer-margin of PAR plus some MFP; **Figure 4**) were excised and fixed in 10% buffered formalin for 24 h, then kept in 70% EtOH until further use. These samples were sent to The Ohio State University Veterinary School to be processed and embedded in paraffin blocks. Upon return to the OARDC, a microtome was used to prepare 5- $\mu$ m thick sections, which were then mounted onto positively charged microscope slides. General immunohistochemistry procedures were carried out as described by Capuco (2007) using a rabbit polyclonal antibody raised against human aromatase as reported by Huderson (2009). Stained microscope slides were observed on an IX81 microscope (Olympus). Five to 10 representative areas of tissue sections were selected at random and digital images were captured at 20x objective magnification using a DP72 camera (Olympus). A representative aromatase stained slide is shown in **Figure 5**.

*Statistical Analysis:* Analysis of variance (SAS software) was used to examine the main effect of twin-type on the dependent variable of aromatase gene expression. Differences were declared significant at  $P \leq 0.05$ . Data are presented as least squares means  $\pm$  standard error of the mean.

## F. Results and Discussion

Aromatase protein was observed in MFP as we thought, but aromatase localization to mammary epithelial cells (MEC) exceeded that of cells which comprise MFP (mainly adipocytes and fibroblasts). For this reason, we investigated aromatase gene expression in mammary parenchyma only. Parenchyma is heterogeneous tissue but contains roughly 20% MEC.

Quantitative reverse transcription PCR compared the level of aromatase present in the total RNA to that of endogenous control gene  $\beta$ -actin. The relative mRNA abundance of aromatase was calculated as the fold difference of the target gene relative to the expression of the endogenous reference genes, as conducted previously in Velayudhan (2008). Regardless of treatment, abundance of aromatase mRNA was low, as evidenced by its late emergence over threshold (>30 cycles; **Figure 6**). As desired, melt curve analysis of aromatase PCR products showed a single peak at 82°C, indicating purity of amplicons and absence of primer dimers (**Figure 7**). Results from quantitative reverse transcription-PCR showed no difference in relative abundance of aromatase due to twin-status ( $P = 0.267$ ; **Figure 8**). Immunohistochemistry data have yet to be analyzed but similar results are expected.

Boesche et al. (2011) highlighted differences in both carcass and mammary gland composition between females with differing twin types (FF vs. FM). We posited that the enzyme aromatase might have been partly responsible for the observed phenotypes and undertook a study to investigate this further. Aromatase is known to be a rate limiting enzyme in the conversion of testosterone to estrogen

The presence of aromatase in prepubertal ovine mammary gland, as demonstrated through both qPCR and antibody staining suggests that the enzyme is present and therefore likely

has a purpose. However, whereas we expected to find more aromatase present in MFP, we actually found it to be more prevalent in PAR. This does not agree with the findings of Huderson (2009), where aromatase gene expression was found to be elevated in MFP of prepubertal dairy heifers. This perhaps shows a species dichotomy in regard to the role of estrogen in prepubertal mammary gland. To that end, it is known that prepubertal sheep respond differently to ovariectomy than do dairy heifers (Ellis et al., 1998; Berry et al., 2003). In prepubertal sheep, ovariectomy does not hinder subsequent PAR growth (Ellis et al., 1998) like it does in prepubertal dairy heifers (Berry et al., 2003). Perhaps our finding of aromatase being more prevalent at the gene level in PAR than in MFP will shed new light onto the role of steroids in mammary gland development. An interesting potential experiment would be to repeat what was done here with the added factor of ovariectomy at an early age in a subset of FM and FF with later comparison to intact cohort groups. This would build on our findings and those of Ellis et al. (1998).

Aromatase is also known to be present and active in the liver of adult sheep. Sharma et al. (2004) declared the liver to be the most important source of aromatase in a male sheep. In two other studies (DeHanan et al., 1987; Jenkins et al., 1988), ewe lambs that were androgenized during gestation had enlarged livers too. Initially it seems as though because the MFP of the FM were enlarged, that perhaps like the liver in previous studies, the enlargement was due to the action of aromatase as it converted testosterone to estrogen. Our findings argue against this though as our gene-level analysis suggested no difference in aromatase abundance due to twin status our preliminary immunohistochemistry findings seemingly agree with this. We do however recognize that lack of difference at the gene level may not necessarily equate to a lack of difference at the protein level. There could be post-translational modifications of the



enzyme that would not be detectable at the qPCR level. Also, it should be pointed out that we did not measure aromatase enzyme activity outright. In retrospect, it would have been interesting to compare the liver weights in these ewe lambs to see if they were different and also to try to measure enzyme activity in the liver and mammary gland using methodology similar to Sharma et al. (2004).

## **G. Conclusion**

Our first objective was to demonstrate aromatase mRNA presence and then quantify relative abundance in snap-frozen mammary tissue samples collected from females of FF and FM twin pairs born to first parity ewes. This objective was completed in PAR but not MFP samples. Total RNA and cDNA yields were low in MFP samples; likewise, aromatase mRNA was below the limit of detection in our qPCR assay for these samples. Aromatase gene was expressed in PAR at low levels and was not different due to twin status. This perhaps could be expected as there were no differences in PAR weight or composition in these ewe lambs (Boesche et al., 2011).

Our second objective was to demonstrate aromatase protein presence and then quantify staining intensity in formalin-fixed, paraffin-embedded mammary tissue samples collected from the same lambs as noted above. Aromatase protein was observed in MFP as we thought, but aromatase localization to mammary epithelial cells (MEC) in PAR was also noted and in many but not all instances, exceeded that of cells which comprise MFP (mainly adipocytes and fibroblasts). This was an interesting finding but the inconsistent staining pattern we observed between animals (data not shown) led us to suspend our immunohistochemical analyses until the staining protocol can be further optimized. As mentioned above, immunohistochemistry data

have yet to be analyzed so objective 2 is not complete at this time. Likewise, because our third objective was to ascertain if the level of expression of the aromatase gene correlates with that of the protein, this too is not complete at this time.

Importantly, we were able to demonstrate aromatase at both the gene and protein levels in ewe lambs, which is a novel finding. This perhaps suggests that aromatase is important for prepubertal mammary growth, though not in the manner we originally hypothesized. Further studies are needed to finish testing our hypothesis, perhaps with different methodology. Given the large disparity in MFP weights in these ewe lambs **Table 1**, it seems plausible that aromatase may still have an impact on MFP even though we could not detect this here.

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**Table 1.** Preliminary mammary and carcass data gathered from offspring of first parity ewes

Item	Twin type		SEM (n = 11)	Twin type <i>P</i> value
	Female / Female	Male / Female		
	(n = 16; 8 sets of twins)	(n = 11)		
Whole Udder, g	143.99	181.20	13.74	0.035
Final BW, kg	46.58	45.99	0.85	0.570
<u>Parenchyma</u>				
Weight, g	20.90	21.50	3.82	0.896
Weight, g/100 kg BW	44.99	47.38	8.49	0.816
Total lipid, g	7.61	7.38	1.85	0.916
<u>Mammary Fat Pad</u>				
Weight, g	123.10	159.70	13.83	0.039
Weight, g/100 kg BW	263.78	346.18	29.83	0.032
Total lipid, g	101.91	131.84	11.80	0.046
KPH fat, kg <sup>a</sup>	0.59	0.47	0.05	0.070
Dress % <sup>b</sup>	56.07	57.87	0.90	0.111
KPH, % <sup>c</sup>	2.25	1.75	0.19	0.040
Average back fat, cm	0.81	0.79	0.09	0.861
Body wall thickness, cm	2.34	2.28	0.08	0.569
Yield grade <sup>d</sup>	3.57	3.50	0.35	0.861

<sup>a</sup> = internal fat taken from around kidneys, pelvic cavity, and heart.

<sup>b</sup> = Hot carcass weight / final weight x 100.

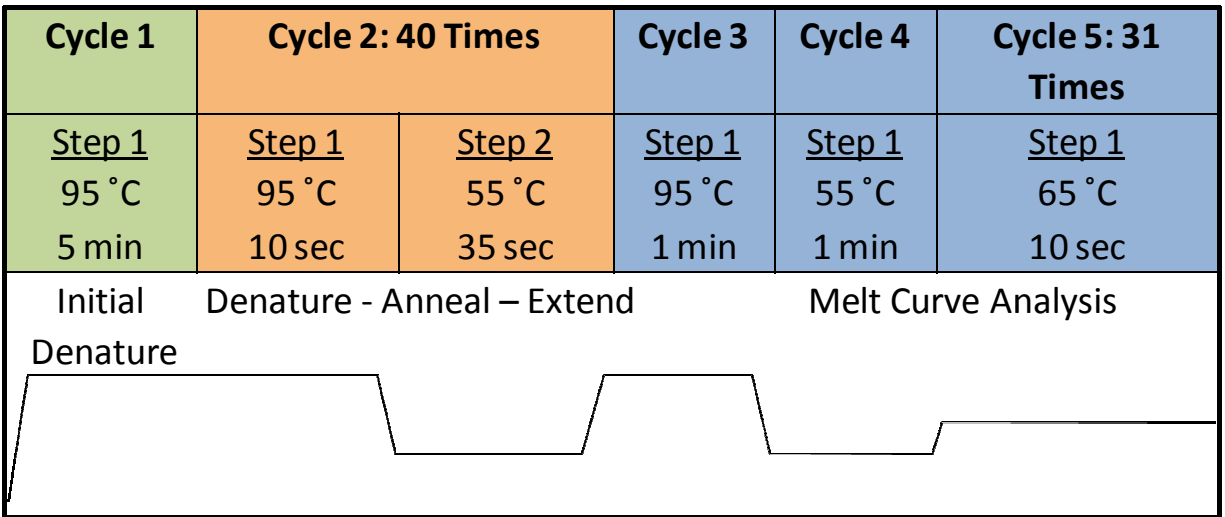
<sup>c</sup> = KPH fat / hot carcass weight x 100.

<sup>d</sup> = Yield grade scores go from 1 to 5.

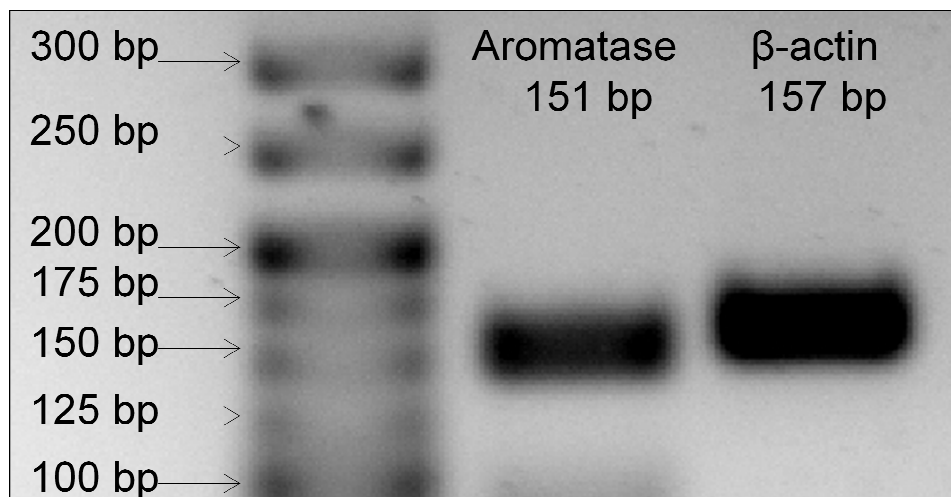
<p align="center"><b>Primer Design</b></p> <p>Aromatase, Ovis Aries (CYP19), Nucleotides – NCBI    <b>1,512 bp</b></p> <p>5'(1)atgcttttg...(854)<b>ccgagaaacgtggtgaactt</b>acaaaagagaatgtaaaccagtgc  atattggaaatgctgatcgacgcccagacaccatgtctgtttctgtgttcttcattgctatttctcattgcaa  agcatccccagggtt<b>gaagagggaatgatgagggga</b>(1004)...ctcgagtgctaa(1521) 3'</p> <p align="center"><b>Product Size = 151 bp</b></p>
--

**Figure 1.** Aromatase primer design within the aromatase gene.

Primer locations are shown in red. Reverse primer for aromatase gene was: TCC-CTC-ATC-ATT-GCC-TCT-TC.

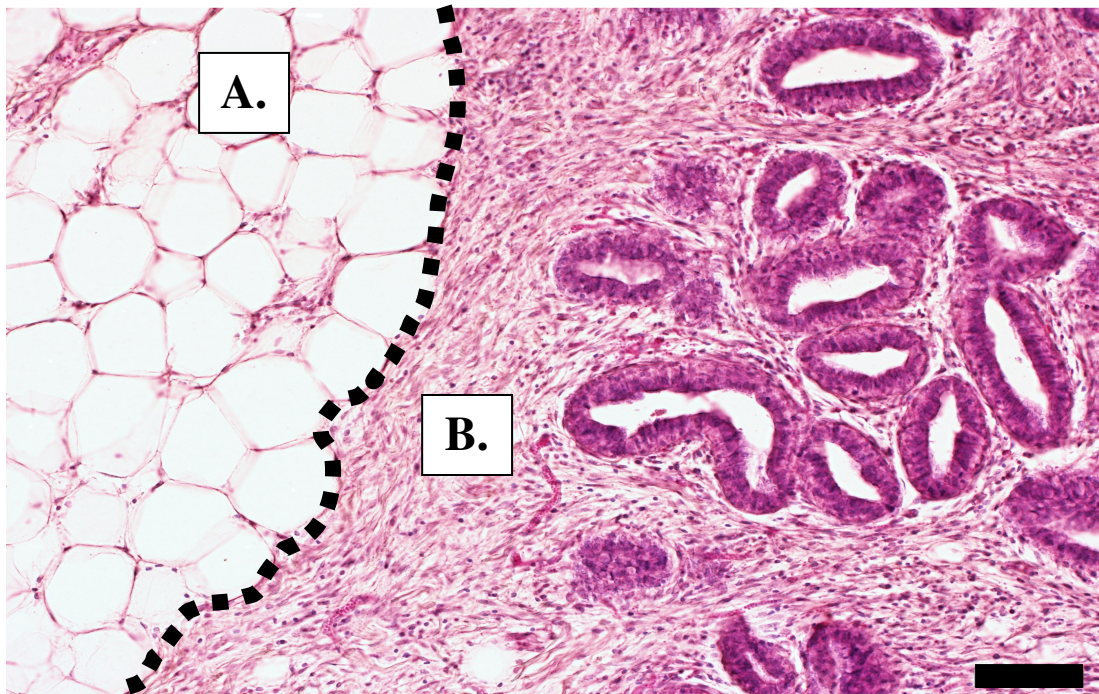


**Figure 2.** Quantitative reverse transcription-PCR settings used.



**Figure 3.** Aromatase and  $\beta$ -actin PCR product size verification.

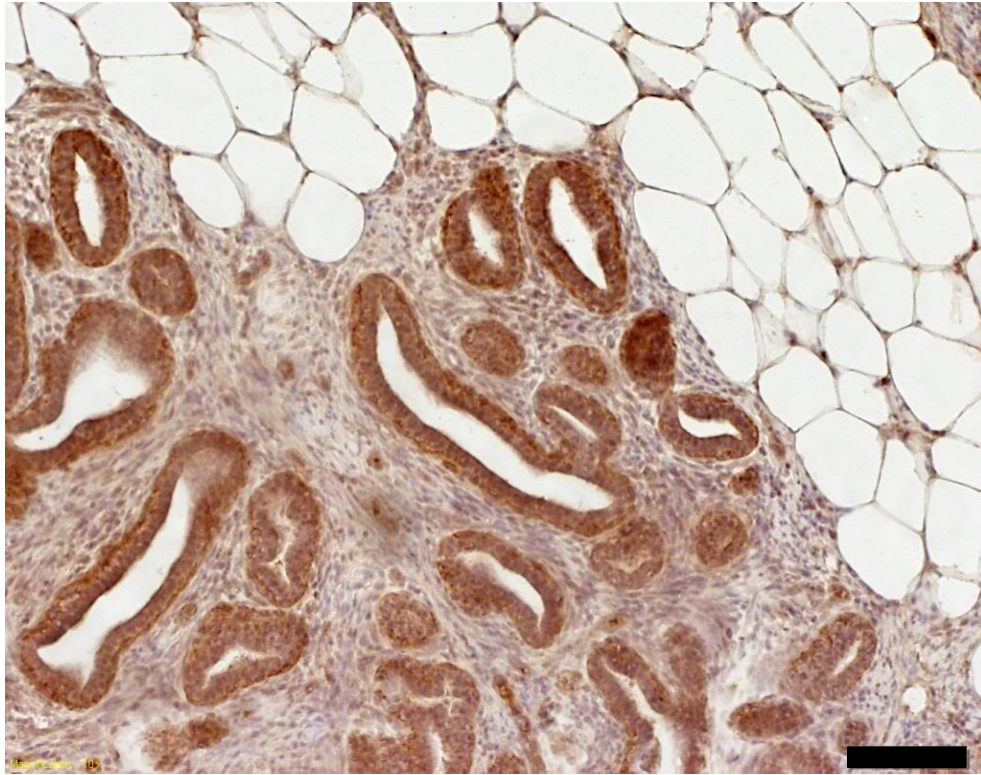
PCR product size was verified via electrophoresis of products and a ladder through a 2% agarose gel.



**Figure 4.** Mammary interface example demonstrating mammary fat pad (MFP) and parenchyma (PAR).

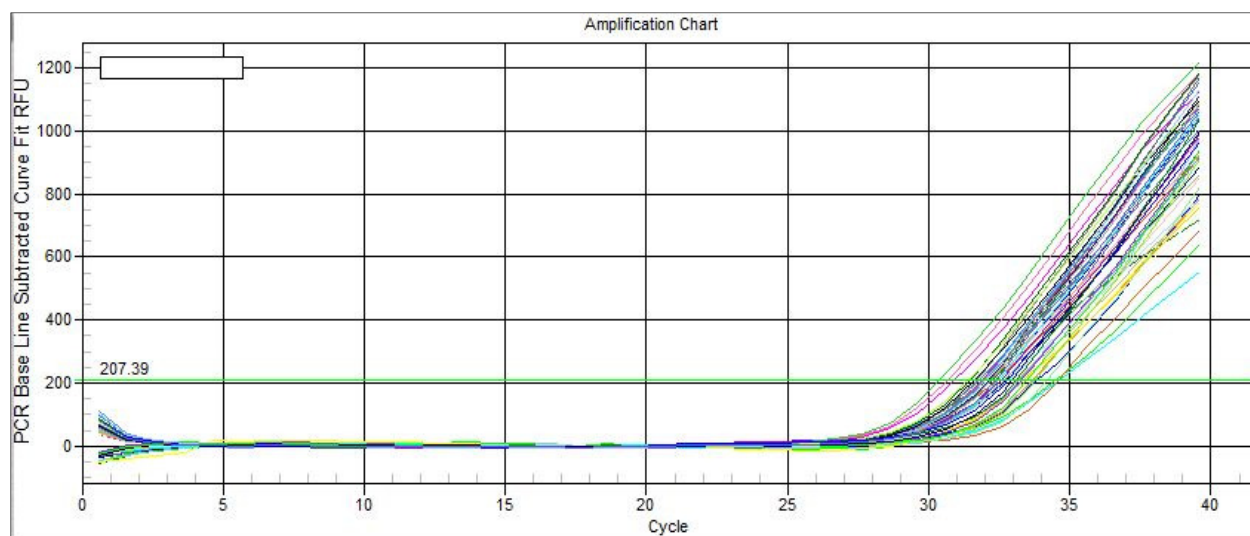
A) illustrates MFP; B) illustrates PAR with epithelial and stromal elements. Dotted line represents demarcation between MFP and PAR. Hematoxylin and eosin stained image shown. Scale bar is 50  $\mu\text{m}$ .





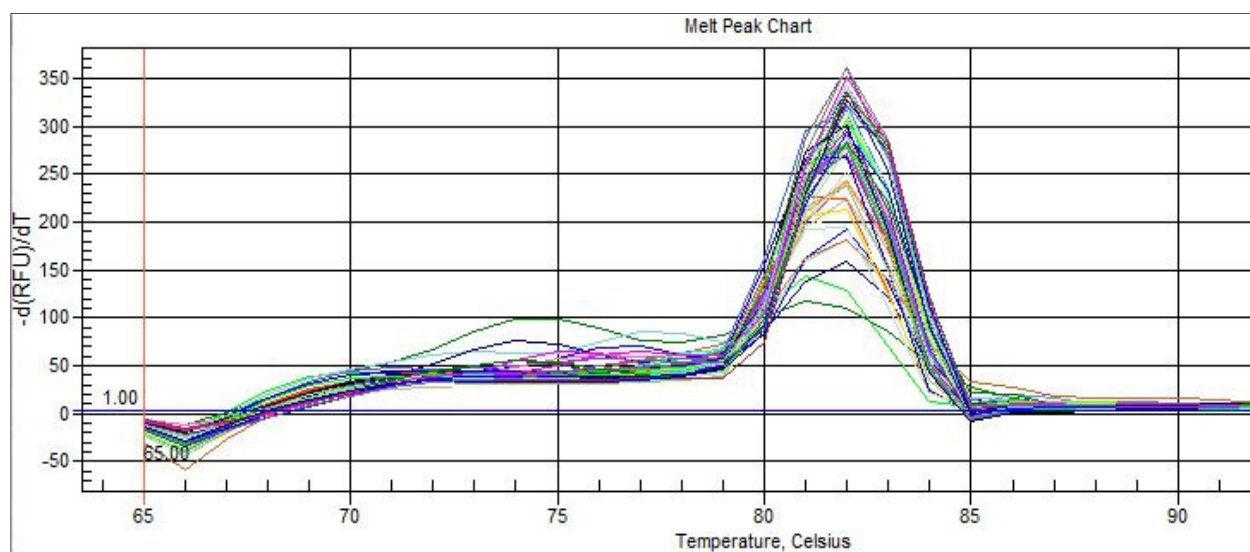
**Figure 5.** Aromatase staining in ovine mammary tissue.

Aromatase antigen (brown stain), hematoxylin counterstain (purple color). Aromatase appears to be localized primarily in epithelial ducts of mammary parenchyma as opposed to adipocytes of mammary fat pad. Scale bar is 50  $\mu\text{m}$ .

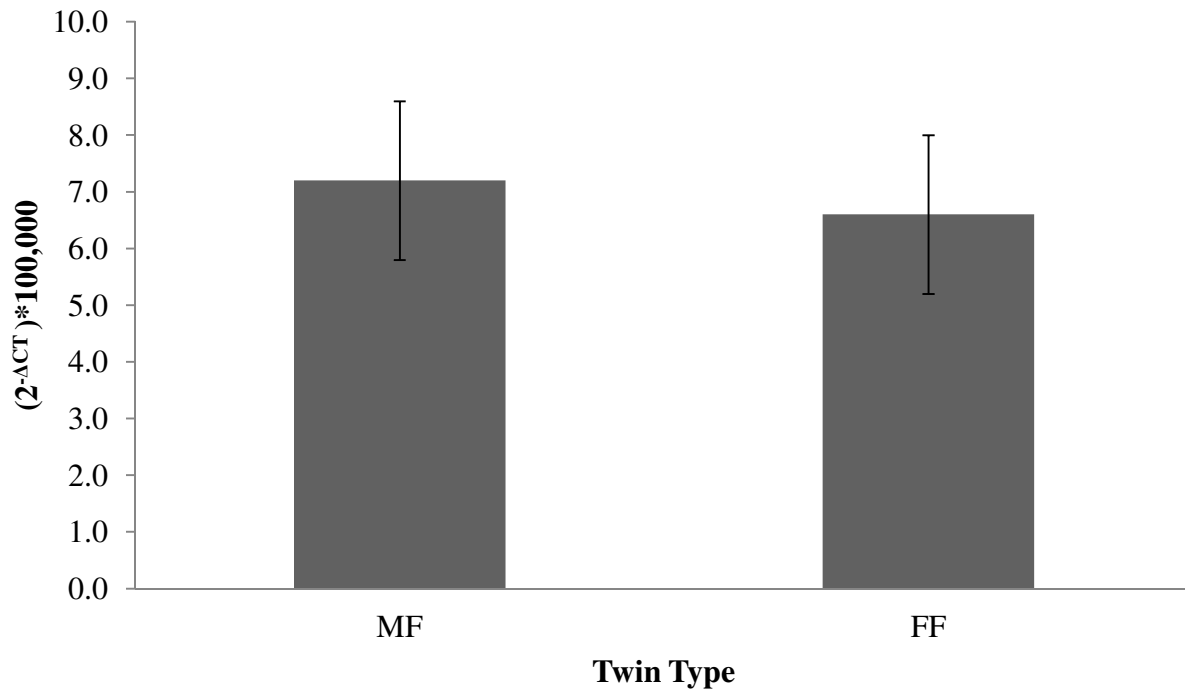


**Figure 6.** Amplification plot for aromatase mRNA in prepubertal ovine mammary parenchyma in a quantitative reverse transcription-PCR experiment.

Regardless of treatment, aromatase mRNA in prepubertal ovine mammary parenchyma was present in small quantities; cycles to threshold exceeded 30 in all cases.



**Figure 7.** Melt curve analysis for aromatase showing a single peak at 82°C.



**Figure 8.** Aromatase mRNA abundance in prepubertal ovine mammary parenchyma.

Data are expressed as  $[(2^{-\Delta C_t}) \times 100,000]$ ; higher values equate to more mRNA. Twin type was either male-female (MF) or female-female (FF). All twins were born to first parity ewes.